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Selective Determination of Melamine in Aqueous Medium by Molecularly Imprinted Solid Phase Extraction

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A molecularly imprinted polymer able to recognize melamine in partially aqueous medium was synthesized using methacrylic acid as functional monomer and ethylene glycol dimethacrylate as cross-linking agent. The bound specificity and selectivity of the obtained material were verified by performing binding experiments with melamine and its structural analogue, 2,4,6-trimethoxy-1,3,5-triazine, respectively, using different aqueous binding media. Finally, the ability of MIP to selectively extract melamine from two real samples, a food supplement and a freeze-dried meat sample, was demonstrated.

KEYWORDS: Melamine; molecular imprinting; solid phase extraction; hydrophilic polymer

INTRODUCTION

Molecularly imprinted polymers (MIP) are synthetic materials owning recognition sites complementary in size and shape for a target molecule called a template (*1*−*3*). The technique to obtain this kind of material involves the formation of complexes between template molecules and functional monomers, followed by polymerization in the presence of a large amount of cross-linker that freezes these template−monomer interactions (*4*,*5*). The subsequent removal of the template results in the formation of a molecularly imprinted polymer matrix (*6*, *7*).

These polymers have found widespread use as adsorbents (*8*), stationary phases for chromatographic separations of molecules (*9*), or ion exchange resins (*10*).

A number of recent publications have shown the effectiveness of the molecularly imprinted solid phase extraction (MISPE) method to determine traces of analytes, particularly in complex pharmaceutical and food samples (*11*, *12*). MISPE is a promising technology that allows the drawbacks of traditional solid phase extraction (SPE) sorbents to be circumvented. The use of MIP as stationary phase in SPE, indeed, displays its main advantage in extractions requiring high selectivity but in which the general sorbents lack selectivity.

Sample processing in SPE normally consists of a nonselective adsorption of the sample onto the cartridge (preconcentration or enrichment), followed by the elution of the analyte(s) of interest in a small volume of an appropriate solvent (*13*). In the case of MISPE, the adsorption of the analytes onto the sorbent is selective, because it occurs on the basis of a specific interaction of the analyte with the polymer matrix. Thus, in the MISPE procedure, if the interfering substances are retained by nonspecific hydrophobic interactions, they are selectively washed and the cleanup of the sample is attained together with the enrichment (*14*, *15*) .

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In this work, a MISPE procedure for the isolation of melamine (ML) in food products was developed.

ML (**Figure 1a**), a trimer of cyanamide, is used to manufacture melamine−formaldehyde resin, a type of plastic known for its flame retardant properties and commonly employed in countertops, dry erase boards, etc. ML can be found at parts per million levels in foods and beverages due to migration from melamine-containing resins or as a metabolite product of cyromazine, an insecticide used on animals and crops.

Due to its nitrogen content, ML is sometimes illegally added to food products to increase their apparent protein content. It has also been employed as a nonprotein nitrogen, appearing in soy meal, corn gluten meal, and cottonseed meal used in cattle feed. Melamine is known to cause renal and urinary problems in humans and animals when it reacts with cyanuric acid inside the body, sometimes present in drinking water and in animal feed, so its use in food production is universally banned.

Health authorities in Europe (European Food Safety Authority, EFSA) and the United States (Food and Drug Administration, FDA) have established a safety limit of 2.5 ppm (ppm) melamine for all food products except infant formula. For milk and infant formula this limit is fixed at 1 ppm melamine. On the basis of these considerations, a reliable method to determine the melamine amount in food samples is needed.

Several methods for ML detection in real samples, such as high-performance liquid chromatography (HPLC) (*16*, *17*), gas chromatography−mass spectrometry (*17*), and capillary electrophoresis (CE) (*18*), have been developed, but all of these protocols involve a very time-consuming procedure for the pretreatment of the samples to concentrate and purify ML for analysis.

In this work, a highly selective sample cleanup procedure combining molecular imprinting and SPE was developed for the isolation of ML in two different food products: a food supplement containing amino acids and vitamins and a freeze-dried meat sample.

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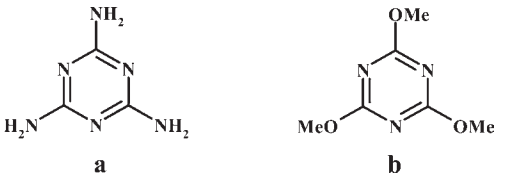

Figure 1. Molecular structures of melamine (**a**) and 2,4,6-trimethoxy-1,3,5-triazine (**b**).

Yang et al. (*19*) developed a MISPE protocol to quantify ML in diary products by using highly cross-linked polymers as sorbent phase and organic solvents in the pretreatment of the samples.

Our goals were to obtain the selective recognition of ML from food matrices in aqueous mixtures without hydrophobic nonspecific interactions and any toxic organic solvent and to show the suitability of the extraction methodology on real samples different from dairy products. Furthermore, our paper reports for the first time the selectivity using a template analogue.

For these purposes, a more hydrophilic polymer was synthesized by increasing the amount of the functional monomer in the prepolymerization feed, and the extraction procedure was applied to two real samples that represent potential and optimal substrates for a contamination with ML due to their high amino acid content.

After the evaluation of the recognition properties of the materials by performing binding experiments in different aqueous media, MISPE cartridges were packed, and their ability to selectively absorb ML was studied by using a structural analogue of ML: 2,4,6-trimethoxy-1,3,5-triazine (TMT, **Figure 1b**). Finally, the ability of the MISPE cartridges to selectively absorb ML from food matrices was investigated. This procedure allows the concentration of the analyte and, after its elution for the cartridges, the immediate analysis of the concentrated eluate by HPLC.

MATERIALS AND METHODS

Reagents and Standards. Ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), 2,2′-azoisobutyronitrile (AIBN), melamine (ML, MW 126.12 g mol^{-1}), and 2,4,6-trimethoxy-1,3,5-triazine (TMT) were obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO). All solvents were of reagent grade or HPLC grade and used without further purification, and they were provided by Fluka Chemika-Biochemika (Buchs, Switzerland).

Preparation of Molecularly Imprinted Polymers. The MIP stationary phase was prepared by bulk polymerization. MAA was used as functional monomer to prepare the MIP by the noncovalent imprinting method. Briefly, 0.5 mmol of template ML, 16 mmol of MAA, 25 mmol of EGDMA, and 0.3 mmol of AIBN were dissolved in 5.5 mL of chloroform in a thick-walled glass tube. The tube was purged with nitrogen, sonicated for 10 min, and then polymerized for 24 h at 60 °C. The resultant bulk rigid polymer was crushed, ground into powder, and sieved through a 63 μm stainless steel sieve. The sieved MIP material was collected, and the very fine powder, suspended in the supernatant solution (acetone), was discarded. The resultant MIP materials were Soxhlet extracted with 200 mL of a methanol/acetic acid (8:2) mixture for at least 48 h. After drying in an oven at 60 °C overnight, the washed MIP polymer was checked to be free of ML and any other compound by HPLC analysis.

Blank polymer (to act as a control) was prepared under the same conditions without using the template.

Binding Experiments. The binding experiments were performed in different ethanol/water mixtures (10:0, 9:1, 7:3, 5:5, 3:7, 0:10 v/v). The polymer particles (20 mg) were mixed with 1 mL of ML solution (0.3 mM) in a 1 mL Eppendorf and sealed. Samples were shaken in a water bath for 24 h and centrifuged for 10 min (10000 rpm), and the ML concentration in the liquid phase was measured by HPLC. In **Table 1**, the amount of ML bound by MIP and NIP samples in each experimental condition is reported.

Table 1. Bound Percentages of Melamine by Imprinted and Nonimprinted Polymers[a]

	ML bound (%)		TMT bound (%)		
binding media	MIP	NIP	MIP	NIP	α value
EtOH (10:0 v/v)	81 ± 0.9	61 ± 1.2	12 ± 0.8	14 ± 1.0	1.33
EtOH/H_2O (9:1 v/v)	67 ± 1.2	53 ± 0.9	11 ± 1.1	15 ± 1.2	1.26
EtOH/H_2O (7:3 v/v)	52 ± 1.1	38 ± 0.8	7 ± 1.3	10 ± 1.3	1.37
EtOH/H_2O (5:5 v/v)	50 ± 0.8	30 ± 1.3	8 ± 1.2	11 ± 1.1	1.67
EtOH/H_2O (3:7 v/v)	46 ± 1.1	53 ± 1.4	15 ± 1.0	20 ± 1.2	0.87
EtOH/H_2O (0:10 v/v)	47 ± 1.0	55 ± 1.1	18 ± 0.9	25 ± 1.2	0.85

[a] Data are shown as means ± SD.

Table 2. Optimization of MISPE Protocol

loading			washing			eluting EtOH/HCl (0.1 M)	
EtOH/H_2O	MIP	NIP	EtOH/HCl (0.01 M)	MIP	NIP	MIP	NIP
5/5	99 ± 0.7	97 ± 1.4	10/0	4 ± 0.5	15 ± 2.0	94 ± 0.9	84 ± 0.9
			9/1	12 ± 0.7	26 ± 1.6	87 ± 1.1	73 ± 0.8
			8/2	20 ± 0.9	65 ± 1.2	80 ± 1.7	35 ± 1.6

The same experiments were performed using TMT solution (**Table 1**), and experiments were repeated five times.

MISPE Conditions. A 500 mg amount of dry particles of polymer was packed into a 6.0 mL polypropylene SPE column. The column was attached with a stopcock and a reservoir at the bottom end and the top end, respectively. The polymer was rinsed with chloroform, acetonitrile, ethanol, an ethanol/HCl 0.1 M mixture (8:2 v/v), water, and then the loading solvent.

To verify the absence of bleeding effect in MIP cartridges, the conditioning solvents were checked to be free of ML and any other compound by HPLC analysis.

ML was dissolved in the loading solvent to a final concentration of 8 μmol/L (1 ppm). After conditioning, a dry MISPE column was loaded with ML standard solution. After column drying, washing solvent was passed through the cartridges, and finally elution solvent was applied to perform the complete extraction of ML. The loading, washing, and eluting fractions were analyzed by HPLC to detect the ML amount. The MISPE protocol was optimized (**Table 2**), and the best conditions were as follows: loading step, 2 mL of ethanol/water mixture (5:5 v/v); washing step, 2 mL of an ethanol/HCl 0.01 M mixture (8:2 v/v); eluting step, 3 mL of an ethanol/HCl 0.1 M mixture (8:2 v/v).

To evaluate the selectivity of the MIP, the same optimized protocol was also applied using a TMT solution, and experiments were repeated five times.

Sample Pretreatment. A food supplement sample containing amino acids and vitamins (500 mg) was crushed, spiked with ML, mixed with 50 mL of a ethanol/water mixture (5:5 v/v), and maintained under magnetic stirring for 2 h. The final ML concentration was 8 μmol/L (1 ppm).

Five hundred milligrams of freeze-dried beef meat was spiked with ML and hydrolyzed with 50 mL of an ethanol/HCl 1 M mixture (5:5 v/v). The sample was then neutralized and used in the MISPE protocol. The final ML concentration was 1 ppm.

MISPE of Food Samples. To test the ability of MIP cartridges to selectively absorb ML from the real samples, the MISPE protocol was performed. Two milliliters of both samples (food supplement and freeze-dried beef meat), spiked at 8 μmol/L (1 ppm) ML, was loaded on the MIP and NIP columns. Thus, the washing step, using 2 mL of an ethanol/HCl (0.01 M) mixture (8:2 v/v), was performed. Finally, 3 mL of an ethanol/HCl (0.1 M) mixture (8:2 v/v) were used as elution fraction.

All of the solutions were analyzed by HPLC, and experiments were repeated five times.

Instrumentation. The liquid chromatograph consisted of a Jasco BIP-I pump and a Jasco UVDEC-100-V detector set at 230 nm. A 25 × 0.4 mm C18 Kromasil column, particle size = 5 μm (Teknocroma, Barcellona, Spain), was employed. The mobile phase was 10 mM

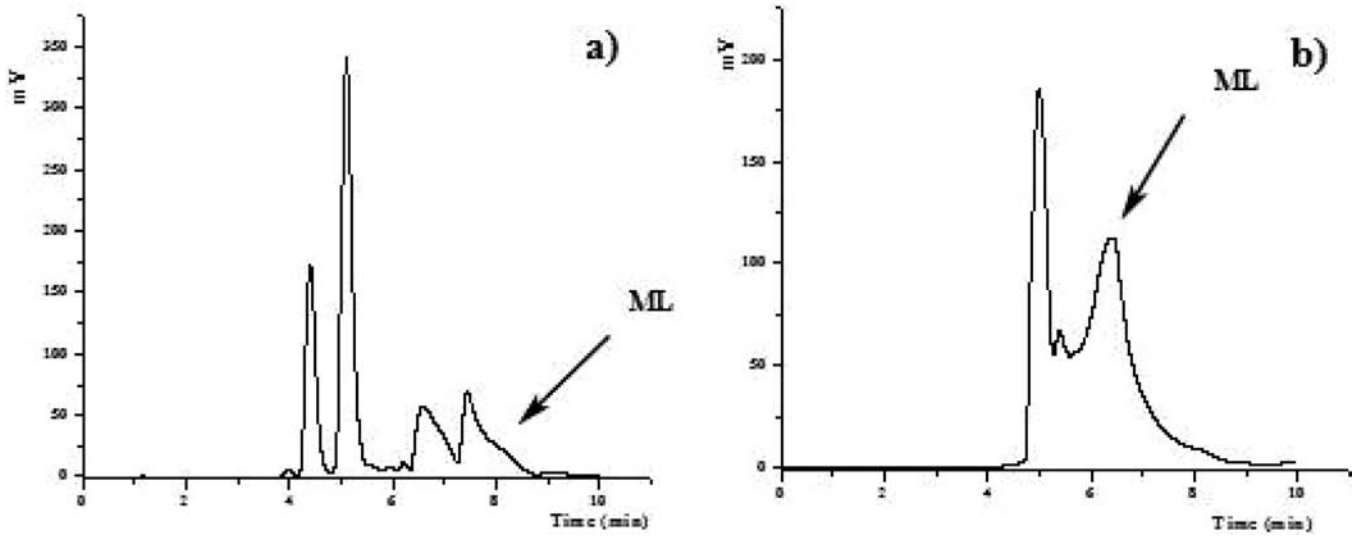

Figure 2. Chromatograms of food supplement (**a**) and freeze-dried meat sample (**b**) after spiking with melamine.

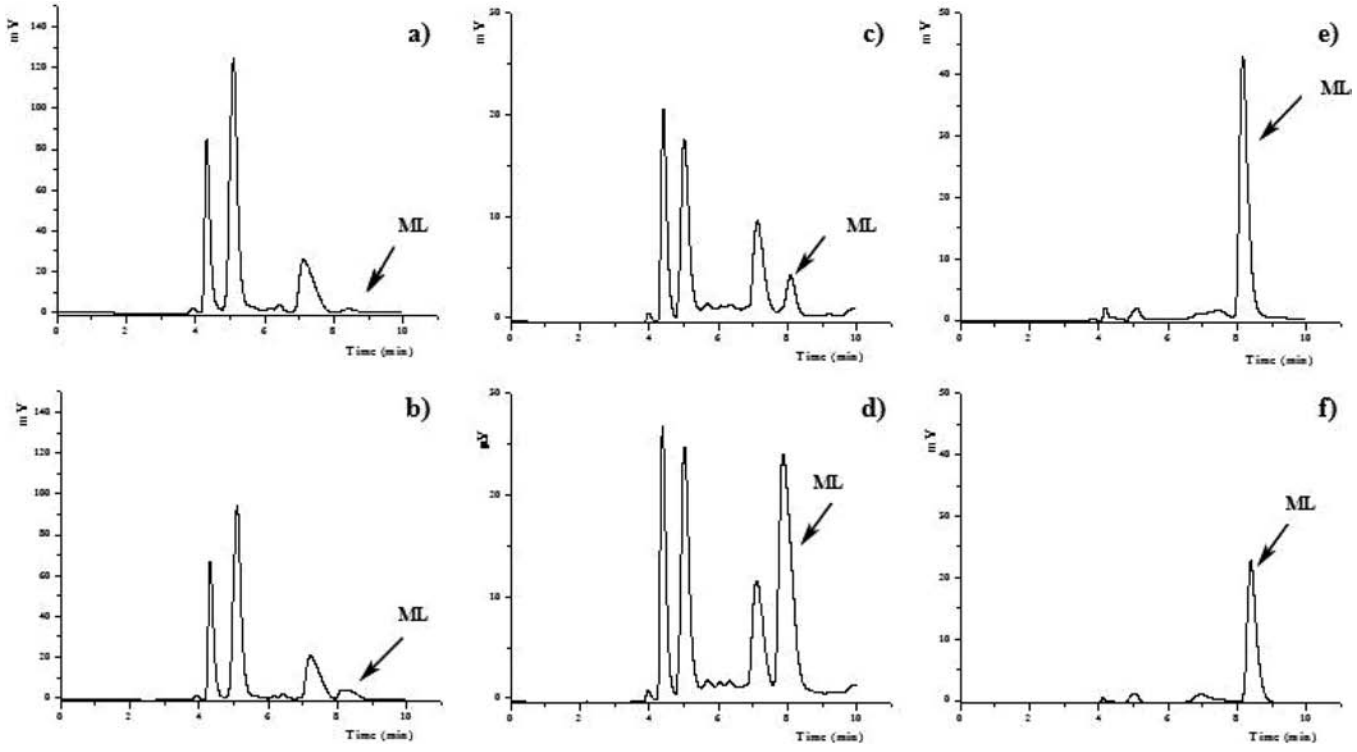

Figure 3. Chromatograms of loading, washing, and eluting fractions of food supplement from MIP (**a**, **c**, **e**, respectively) and NIP (**b**, **d**, **f**, respectively) cartridges.

sodium *n*-heptanesulfonate/acetonitrile (83:17, v/v, pH 2.7) (*20*), eluted isocratically at a flow rate of 0.5 mL/min.

The shaker and centrifugation systems consisted of a wrist action shaker (Burrell Scientific) and an ALC microcentrifugette 4214, respectively.

Analytical Parameters. The calibration curve and detection and quantitation limits (LOD and LOQ, respectively) were determined using ML-spiked real samples. Real samples (0.050 g) were spiked with 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.54, or 5.08 mg of ML and extracted with 50 mL of the respective extraction solvents, and the MISPE protocol was performed. Detection and quantification limits were calculated as the ML concentration in the MISPE eluting fractions corresponding to a signal 3 and 10 times, respectively, the standard deviation of the baseline noise (*21*). To correlate these values with the MISPE applicability to the real samples, LOD and LOQ were then expressed as the relative ML concentration in the starting spiked food matrices.

The repeatability of the MISPE method was evaluated by performing five repetitive analyses on five different samples of the same food matrix. The intraday and interday precisions of the relative peak areas were calculated as RSDs for five measurements.

RESULTS AND DISCUSSION

Synthesis and Characterization of Imprinted Polymers. ML-imprinted polymer was synthesized by a noncovalent approach using MAA as functional monomer and EGDA as cross-linking agent.

Because our goal was to obtain an imprinted polymer able to recognize ML in an aqueous environment (ethanol/water mixture), in the polymerization feed a high amount of functional monomer was employed. Water and ethanol, indeed, are hydrogen-bonding

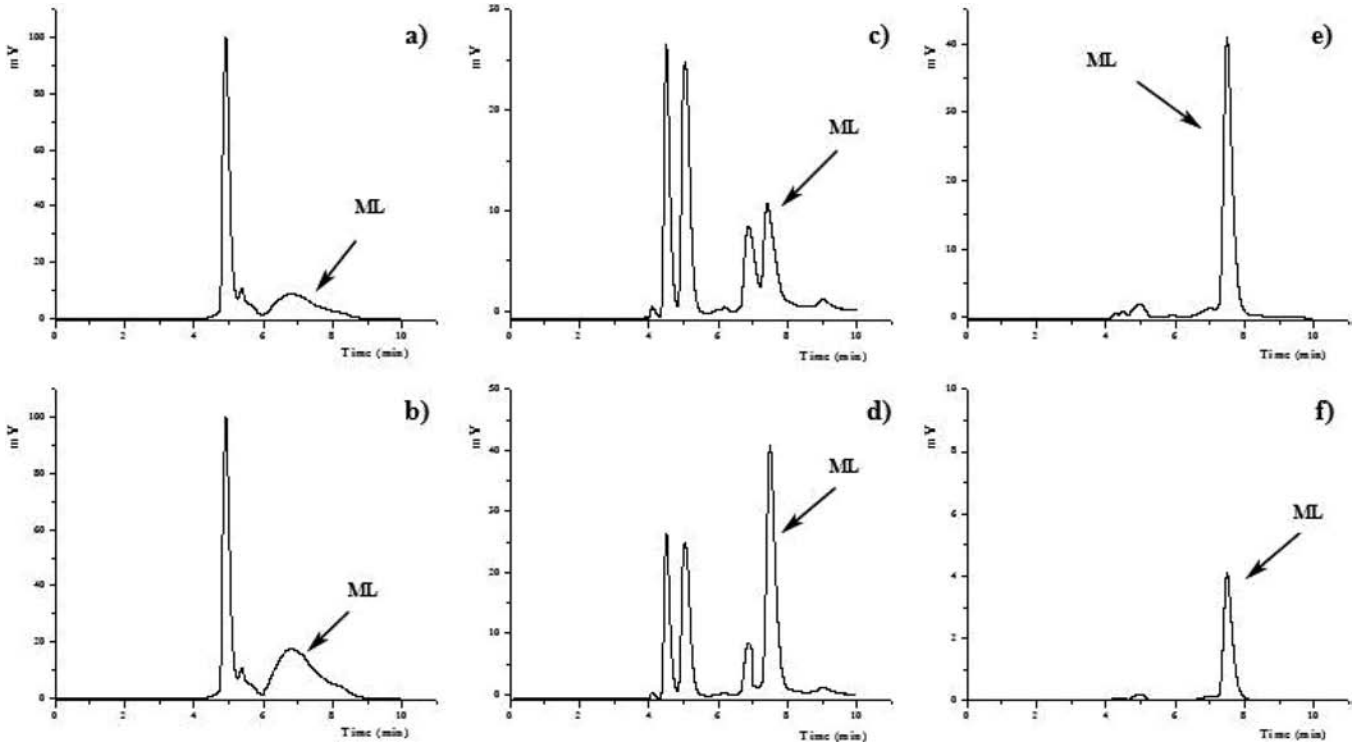

Figure 4. Chromatograms of loading, washing, and eluting fractions of freeze-dried meat sample from MIP (**a**, **c**, **e**, respectively) and NIP (**b**, **d**, **f**, respectively) cartridges.

disruptors, and thus, to drive the binding equilibrium toward the polymer−template assembly, an increase of the classical functional monomer/template molar ratio is required. Furthermore, in this way, compared to previous research work (*11*), MIP with increased hydrophilicity and thus better imprinting efficiency, due to reduction of nonselective hydrophobic interactions, can be obtained.

The imprinting effect of MIP was evaluated by performing binding experiments in which amounts of polymeric particles were mixed with template solutions. In particular, as before mentioned, different ethanol/water mixtures were employed to verify the hydrophilic characteristics of our material. The obtained results, reported in **Table 1**, show that the ML bound specificity increases progressively as the water content in the rebinding media increases, reaching the best value in the ethanol/water (5:5 v/v) mixture, as a confirmation of the hydrophilic properties of MIP. On the other hand, a further increase of the water percentage in the rebinding media results in a lower imprinting efficiency due to the predominance of aspecific ionic interactions between the template and the polymer functionalities. In **Table 1**, the imprinting efficiency was numerically expressed using α values, which were calculated as the ratio of bound template (%) by MIP and NIP (*12*). The α value is a measurement of the binding specificity and, thus, a high α value indicates a higher performing material.

As well as the bound specificity, a primary and indispensable requirement of MIP is the selectivity of the binding cavities toward the template.

Thus, the selectivity properties of MIP were evaluated by performing the same binding experiments using TMT, a structural analogue of ML. As shown in **Table 1**, the TMT binding percentages are much lower than that recorded for the template, confirming the selectivity of the imprinted sites.

MISPE of ML and TMT Standard Solutions. After the evaluation of the imprinting efficiency, the applicability of ML-imprinted polymer as stationary phase in the SPE procedure was investigated.

MISPE is based on conventional SPE procedures; therefore, conditioning, loading, cleanup, and elution steps are performed as a matter of routine.

The loading step was performed using a ML standard solution in an ethanol/water (5:5 v/v) mixture at a flow rate of 0.2 mL min^{-1}, obtaining a complete retention of the template in both MIP and NIP cartridges. This loading mixture was selected because of the highest imprinting effect, as shown by the recorded α value (**Table 1**).

Thus, to minimize the aspecific component of the interaction between ML and polymeric matrices, a washing step was performed using an ethanol/HCl 0.01 M (8:2 v/v) mixture. In this condition, 20% of ML was recovered in the washing fraction of MIP cartridges, whereas in NIP cartridges this percentage increased to 65%, because of the absence of specific interactions between the template and the polymeric materials.

The optimized elution was raised by employing an ethanol/HCl 0.1 M (8:2 v/v) mixture, obtaining a recovery of ML equal to 80% for MIP cartridges, whereas in NIP cartridges ML was only 35%.

The selectivity of the packing cartridges was evaluated by using TMT solution in the same conditions tested for ML. In this case, a complete loading of the TMT standard solution was raised in both MIP and NIP cartridges, but only in the washing fraction was a complete recovery of the analyte obtained.

MISPE on Real Samples. The previous SPE protocol was carried out to obtain the isolation of ML from two real products: a food supplement containing amino acids and vitamins and a freeze-dried meat sample. The food supplement sample was crushed, spiked with ML, and then extracted in the loading mixture. For the freeze-dried meat sample, instead, the pretreatment involved the spiking with ML followed by a hydrolytic step, using an ethanol/HCl (0.1 N) mixture (5:5 v/v), to hydrolyze the proteic fraction of the sample, and neutralization. In both samples, the final ML concentration was 8 μmol/L (1 ppm), which represents the lowest limit imposed by FDA regulations.

In **Figure 2a**, the HPLC chromatogram of the food supplement sample after spike and pretreatment is reported.

The sample was loaded onto MISPE cartridges, obtaining a ML retention percentage equal to 95% (**Figure 3a**), whereas, for the NIP cartridge, this percentage decreased to 87%.

The washing step, performed with an ethanol/HCl (0.01M) mixture (8:2 v/v), allowed a good cleanup of the matrix. In **Figure 3c**, which is the chromatogram of the MISPE washing fraction, several peaks referable to other components of the food samples are shown, but at the retention time of ML, a low percentage of the loaded ML was recorded (10%). When the same washing mixture is loaded on the NIP cartridge, instead, a considerably higher ML percentage (45%) is recovered.

The subsequent elution step (**Figure 3e**), performed with an ethanol/HCl (0.1 M) mixture (8:2 v/v), achieved a complete and selective recovery of ML (85%).

The chromatogram of freeze-dried meat sample after spike shows a coelution of ML with the other components of real sample (**Figure 2b**). Thus, to obtain the purification of ML from the other components of the mixture, the same SPE conditions employed for the first sample were applied, obtaining, in the elution fraction, ML recovery percentages of 77% (**Figure 4e**) for the MIP cartridge and 7% for the NIP cartridge (**Figure 4f**).

For the food supplement, LOD and LOQ values correspond to a ML concentration in the loading fraction of 6.82×10^{-7} mol L^{-1} (8.66×10^{-5} g L^{-1}) and 1.56×10^{-6} mol L^{-1} (1.97×10^{-4} g L^{-1}), respectively, whereas for the freeze-dried meat these values are 7.12×10^{-7} mol L^{-1} (8.97×10^{-5} g L^{-1}) and 1.73×10^{-6} mol L^{-1} (2.18×10^{-4} g L^{-1}). The calibration curves were linear for both the food supplement (ranging from 6.82×10^{-7} to 8.3×10^{-4} mol L^{-1}; $R^2 = 0.9978$) and the freeze-dried meat (ranging from 1.56×10^{-6} to 8.9×10^{-4} mol L^{-1}; $R^2 = 0.9962$).

The intraday precisions of the relative peak areas were between 2.7 and 3.6% for the food supplement and between 3.1 and 4.3% for the freeze-dried meat; the interday precisions were between 7.2 and 8.6% for the food supplement and between 7.7 and 9.4% for the freeze-dried meat.

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